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ROLE OF INTRACELLULAR THIOL STATUS AND CALCIUM HOMEOSTASIS
IN MYOCARDIAL CELL INJURY
BY
CYRUS M. DHANBHOORA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN PHARMACOLOGY AND TOXICOLOGY

UNIVERSITY OF RHODE ISLAND

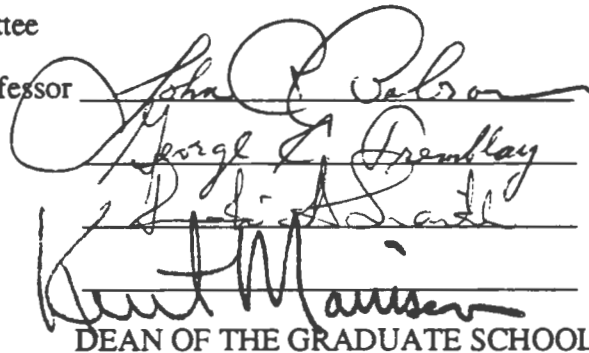
1990

MASTER OF SCIENCE THESIS
OF
CYRUS M. DHANBHOORA

APPROVED:

Thesis Committee

Major Professor


DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

1990

ABSTRACT

The primary biochemical mechanisms involved in chemically-induced cell injury remain to be elucidated. Elevation of intracellular Ca^{2+} is a common feature to cell death due to a wide array of toxic chemicals, and on this basis hypotheses have been put forth suggesting that the chemically-induced elevation of cytosolic Ca^{2+} is responsible for the onset of cell death. The mechanism by which elevated Ca^{2+} causes cell damage may involve activation of Ca^{2+} -dependent proteases, phospholipases and endonucleases (2). Previous evidence suggests that a link between intracellular thiol status and Ca^{2+} homeostasis exists (1, 2). Based on these observations, it has been speculated that thiol depletion may lead to an elevation of intracellular Ca^{2+} to cytotoxic levels (1, 2, 4).

Glutathione, the major cellular thiol, is primarily a defense mechanism against cytotoxic reaction to oxidative stress or alkylating agents. Glutathione also plays an important role in maintaining protein thiols in a reduced state, which is required for their normal enzymatic activity (6, 7). Such enzymes include the sarcolemmal and sarcoplasmic reticular Ca^{2+} -ATPase's, which are involved in the maintenance of low levels of cytosolic Ca^{2+} . Therefore depletion of intracellular glutathione may limit the capacity of these enzymes with modified thiol groups to maintain low levels of cytosolic Ca^{2+} (10, 11). The loss of GSH as an antioxidant may promote oxidative stress and the resultant peroxidative damage to plasma membrane may be an alternate cause of cell death by a Ca^{2+} -independent mechanism. The relative importance of elevated cytosolic free Ca^{2+} or oxidative stress in cell death, in the face of a chemical challenge that alters intracellular thiol status is the subject of this thesis.

Our approach towards this problem was to create a chemical model of oxidative stress in cardiomyocytes using ethacrynic acid. Ethacrynic acid depletes thiols by alkylation with a subsequent increase in cytosolic free Ca^{2+} , thereby permitting us to examine lethal cell injury due to thiol depletion, including the proposed link to Ca^{2+} homeostasis. Exposure

of primary rat myocardial cells to ethacrynic acid (150 μ M) resulted in a rapid (within 7 min) loss of glutathione and protein thiols that preceded an increase in cytosolic free Ca^{2+} levels (within 45 min), as detected by the activation of phosphorylase *a*. The leakage of cytosolic lactate dehydrogenase due to loss of membrane integrity was used as a criterion of loss of cell viability. All of these biochemical events preceded the loss of cell viability, thus permitting us to examine whether thiol depletion or changes in cytosolic free Ca^{2+} had the primary effect on the loss of cell viability.

Pretreatment of cells with specific intracellular Ca^{2+} chelators, Quin-2-acetoxymethylester and EGTA-acetoxymethylester, were used in an attempt to sequester Ca^{2+} , in order to prevent an ethacrynic acid-induced elevation of intracellular Ca^{2+} . Both intracellular chelators reduced lactate dehydrogenase leakage, protected against lipid peroxidation, but failed to reduce the marked elevation of intracellular Ca^{2+} . The latter observation required examination of the mechanism of protection afforded by the putative chelators. The antioxidant N,N'-Diphenyl-p-phenylenediamine was employed to investigate the importance of lipid peroxidation in ethacrynic acid-induced cell death. N,N'-Diphenyl-p-phenylenediamine reduced lipid peroxidation and lethal cell injury to control levels but had no effect on intracellular glutathione and Ca^{2+} levels. Thus, it would appear that the antioxidant activity of the putative chelators might account for their protection. The possibility that cytotoxicity was due to an ethacrynic acid-induced alteration of cellular energy status was also examined. Ethacrynic acid had no significant effect on cellular ATP levels or mitochondrial membrane potential.

In our model of myocardial cell injury the temporal relationship observed between the loss of intracellular thiol status and Ca^{2+} homeostasis supports the hypothesis that thiol status is linked to Ca^{2+} . However elevated Ca^{2+} levels alone, had no effect on cell viability over the time course we observed, further supporting that peroxidative damage is a requisite event for cell death in our model of myocardial cell injury.

ACKNOWLEDGEMENT

The author would like to thank Dr. John R. Babson for his utmost patience, moral support, and guidance throughout the course of this study.

I would like to dedicate this thesis in loving memory of my uncle Dr. Rusi K. Dastoor, who unknowingly helped mold my career and outlook to life. I would also like to dedicate this work to my parents and my girlfriend Pia for their undying love and support. Without their love, support, and understanding, this work would not have been completed. Finally, I would like to thank special friends Michael Christie, Mark Blazka, Gunturi Srinivas, and Nancy Gavitt for all their help.

PREFACE

This thesis was prepared according to the manuscript format. The manuscript will be submitted to Biochemical Pharmacology.

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**ROLE OF INTRACELLULAR THIOL STATUS AND CALCIUM
HOMEOSTASIS IN MYOCARDIAL CELL INJURY**

ABSTRACT

Ethacrynic acid, was used to deplete intracellular thiols to create a model of oxidative stress in order to examine the ensuing events leading to myocardial cell injury. Exposure of primary rat myocardial cells to ethacrynic acid resulted in a rapid loss of glutathione and protein thiols that preceded an increase in cytosolic free Ca^{2+} levels, as detected by the activation of phosphorylase α . The magnitude of lethal cell injury, using leakage of lactate dehydrogenase as a criterion, was dependent on the ethacrynic acid concentration used. The loss of cellular thiols and the elevation of intracellular Ca^{2+} preceded the onset of cell death. Pretreatment of cells with specific intracellular Ca^{2+} chelators, Quin-2- acetoxymethylester and EGTA- acetoxymethylester, were used in an attempt to sequester Ca^{2+} and thereby prevent an ethacrynic acid-induced elevation of intracellular Ca^{2+} , in this model system of chemically-induced cell killing. Both intracellular chelators reduced leakage of lactate dehydrogenase, protected against lipid peroxidation, but failed to reduce the marked elevation of intracellular Ca^{2+} . The latter observation required examination of the mechanism of protection afforded by the putative chelators. The antioxidant N,N'-Diphenyl-p-phenylenediamine was employed to investigate the importance of lipid peroxidation in ethacrynic acid-induced cell death. N,N'-Diphenyl-p- phenylenediamine reduced lipid peroxidation and lethal cell injury to control levels but had no effect on intracellular glutathione and Ca^{2+} levels. Thus, one could postulate that the antioxidant activity of the putative chelators might account for their protective properties. An alternative possibility that cytotoxicity was due to an ethacrynic acid-induced alteration of cellular energy status was also examined. Ethacrynic acid had no significant effect on cellular ATP levels or release of the triphenylmethylphosphonium cation, a measure of mitochondrial membrane potential. These results support previous observations that a loss of intracellular thiols is followed by a rise in Ca^{2+} , and the

perturbation of these homeostases may result in the loss of cell viability. However the elevated Ca^{2+} alone was not responsible for cell death over the time course we observed, and oxidative damage was seen to be a primary requisite for myocardial cell injury.

INTRODUCTION

An elevation of intracellular Ca^{2+} is a response that is frequently observed following exposure to a wide variety of toxic chemicals (1). This observation led to the hypothesis that chemically-induced elevation of cytosolic Ca^{2+} is a causative event in the cytotoxic mechanism of many chemicals (2). Attempts to understand how chemicals perturb intracellular Ca^{2+} homeostasis have provided evidence that suggests the elevation of cytosolic Ca^{2+} is a direct result of thiol depletion (1, 2). However, it may be the oxidative stress that results from thiol depletion, and not the accompanying rise in Ca^{2+} , that is responsible for cell death (2,4, 6). Based on all of these observations, two different mechanisms have been proposed to explain chemically-induced cell injury: 1. A Ca^{2+} -dependent mechanism of cytotoxicity involving a perturbation of Ca^{2+} homeostasis that triggers Ca^{2+} -activated processes that are ultimately responsible for cell death. 2. A Ca^{2+} -independent mechanism in which cell death is caused by peroxidative damage that results from the loss of glutathione (GSH) as an antioxidant.

GSH, a thiol tripeptide, is a major component of intracellular thiol status and is also a primary cellular defense mechanism against toxic chemical insult (1, 2, 4). GSH is distributed intracellularly in two main pools. The mitochondrial pool contains 15% and the cytosolic pool contains 85% of the total intracellular GSH. GSH protects the cell against toxic challenges via two distinct mechanisms. First, it detoxifies alkylating agents by its ability to form conjugates either directly or enzymatically via GSH-S-transferases (8, 9). Second, GSH protects cells against oxidative stress by its ability to reduce chemical oxidants through the GSH redox cycle (6). Some studies have suggested that the depletion of mitochondrial GSH is a key factor in the onset of cell death (7).

GSH is also involved in the normal functioning of cells, including the maintenance of protein thiol status (6). The reducing equivalents provided by GSH maintain the activity of

many cellular enzymes, which require that certain protein thiols be in the reduced state for enzymatic activity (10). Included in this group of enzymes are the sarcolemmal and sarcoplasmic reticular Ca^{2+} -ATPase's believed to be involved in the sequestration and extrusion of cytosolic free Ca^{2+} (10, 11). Since protein thiols may also be alkylated by toxic chemicals, GSH provides a nucleophilic barrier that protects protein thiols from alkylation. Accordingly, any event resulting in GSH depletion may lead to an alteration of key enzyme thiols, and subsequently limit the capacity of these enzymes to regulate intracellular Ca^{2+} levels (7).

Under normal physiological conditions the cytosolic free Ca^{2+} concentrations are quite low, ranging between 50 and 200 nM (14). It has been proposed that many toxic chemicals may elevate cytosolic free Ca^{2+} by a combination of two processes. First, they are believed to promote the release of intracellular Ca^{2+} stores or the influx of extracellular Ca^{2+} by mechanisms that have yet to be determined. Second, by inhibiting Ca^{2+} -ATPase activities responsible for removing Ca^{2+} from the cytosol, the chemical challenges are believed to elevate cytosolic free Ca^{2+} (12, 15, 24). Whether extracellular or intracellular Ca^{2+} is the primary source of elevated cytosolic free Ca^{2+} observed in lethal cell injury (18, 19), is beyond the scope of this study. Regardless of the source or mechanism, any chemical insult that promotes a sustained elevation of cytosolic Ca^{2+} , including those that deplete intracellular thiols, may ultimately cause lethal cell injury due to a variety of Ca^{2+} activated processes (2) . Elevated cytosolic Ca^{2+} could be expected to activate several Ca^{2+} -dependent enzymes, including Ca^{2+} -activated proteases, Ca^{2+} -activated phospholipases, and Ca^{2+} -activated endonucleases (1, 2, 20). Prolonged activation of these enzymes is believed to contribute to cell death (1, 3).

A number of studies conducted on hepatocytes, and some on myocardial cells, suggest that cell injury due to thiol depletion results from the consequent elevation of intracellular Ca^{2+} (1, 2, 25). However, there is equally persuasive evidence to suggest

that thiol depletion alone may cause cell death, by a Ca^{2+} -independent mechanism, through resultant oxidative damage (16, 17). Although lethal cell injury accompanying oxidative stress is associated with elevation in cytosolic free Ca^{2+} , the loss of cell viability may not result from the observed changes in intracellular Ca^{2+} levels (32). The loss of GSH as an antioxidant may play a more important role in loss of cell viability, through oxidative damage, and the resultant lipid peroxidation (3, 21).

In this study our approach towards this problem was to create a model of oxidative stress, using ethacrynic acid (EA), a selective sulfhydryl alkylating agent (3, 40, 41), to deplete intracellular thiols. This model permitted us to examine the key biochemical events affected by thiol depletion, including perturbations of Ca^{2+} homeostasis, and the contribution of these events to cell death. A major goal of this study was directed towards attempting to elucidate the role of thiol status in chemically-induced lethal cell injury and to determine the relative importance of elevated intracellular Ca^{2+} levels and oxidative stress to cell death.

METHODS

Cardiomyocyte Isolation and Culturing. Myocardial cells were isolated and cultured essentially according to the method of Bollon et al (26) with several modifications aimed at increasing the yield of beating myocytes over that of non-muscle cells (27-29). Cells were plated in separate 35 mm culture dishes, incubated in Eagles MEM medium buffered with 25 mM Hepes, pH 7.4 and containing 10% horse serum, 5% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 U/mL insulin at 37°C and 95% humidity . Cells were used on the eighth day after initial plating. Beating rate provides an indicator of the quality of preparations. Cells used throughout this study had an average beating rate of between 140-160 beats/min .

General Conditions for Exposure to Chemicals and Solvents. Cells were rinsed twice with Hank's balanced salt solution containing 25 mM Hepes, pH 7.4, and incubated at 37°C with 150 µM EA, in the absence or presence of 5 µM N,N'-Diphenyl-p-phenylenediamine (DPPD), 120 µM EGTA-acetoxymethylester (EGTA-AM) or 120 µM Quin-2-acetoxymethylester (Quin-2-AM) for the stipulated incubation time (7-240 minutes). Appropriate solvent controls were conducted for all experimental conditions. Solvent controls for all EA incubations contained 0.3% (v/v) ethanol. Controls for cells treated with DPPD contained 0.5% (v/v) DMSO, in addition to 0.3% ethanol. For studies using Quin-2-AM or EGTA-AM, the cells were preincubated with 120 µM Quin-2-AM or 120 µM EGTA-AM, dissolved in DMSO (0.4-0.6%, v/v) for 45 min at 37° C. At the end of incubation time the plates were rinsed twice with Hank's balanced salt solution and incubated in the presence or absence of 150 µM EA for the appropriate time. Controls for Quin-2-AM or EGTA-AM were preincubated with 0.4-0.6% DMSO (v/v), then incubated in the presence or absence of EA. At the end of the incubation time, the incubation solutions were removed from the plates and retained for use in the biochemical

determinations mentioned below. Adherent myocardial cells were lysed with lysing buffer containing 100 mM NaF, 20 mM EDTA, 50 mM glycylglycine, 0.5% Triton x-100, and 0.5% glycogen, pH 7.4, according to the procedure of Long and Moore (30). Cells were scraped from plates, centrifuged at 13,500g for 5 minutes, and the resultant supernatant, termed cell lysate, was removed and reserved to obtain the following biochemical measurements.

Biochemical Assays.

Lactate Dehydrogenase (LDH) Activity. LDH leakage into the incubation medium was used as an indicator of cell death. LDH activity was measured by monitoring the disappearance of NADH at 340 nm, spectrophotometrically by the method of Lindstrom et al (31) and expressed as a percentage of total cellular LDH.

Phosphorylase a Assay. The Ca^{2+} -dependent conversion of phosphorylase *b* to phosphorylase *a* was used as an indirect estimate of intracellular free Ca^{2+} . Phosphorylase *a* was determined by measuring the Pi released from glucose-1-phosphate, by the method of Gilboe et al (33), as modified by Farber et al (32).

Phosphatidylinositol Hydrolysis. An alternate indirect measurement of intracellular Ca^{2+} was conducted by measuring the phosphatidylinositol hydrolysis by Ca^{2+} -activated phospholipase *c*. The assay procedure of Slivka et al (34) was followed. Cellular phosphatidylinositol was radioabeled by the incorporation of 2 $\mu\text{Ci/mL}$ of [2- ^3H]-*myo*-inositol for 24 hr at 37°C. Following various chemical exposures, the cells were precipitated with 10% trichloroacetic acid (TCA) and the resultant supernatant were analyzed for the radiolabel released into the medium. The pellets were solubilised in 5 ml scintillation fluid and analyzed for the radiolabel retained. Results were expressed as the percent of total [2- ^3H]-*myo*-inositol hydrolysed.

Protein Thiol and GSH Analyses. The original method of Sedlak and Lindsay (35), as

modified by Orrenius et al (36) was followed. Briefly, cell incubate was removed and plates were treated with 6.5% TCA, scraped and centrifuged at low speed, and the resultant supernatants analyzed for GSH levels by the HPLC method of Reed et al (37). To estimate protein thiols, the pellet was washed twice with 6.5% TCA and suspended in 0.5M Tris-HCl containing 100 μ M 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), pH 8.3, and the absorbance read at 412 nm. Results were expressed as nanomoles SH/mg protein.

Lipid Peroxidation Assay. Lipid peroxidation was measured by monitoring the formation of a colored complex between malondialdehyde and thiobarbituric acid (TBA), by the method of Stacey and Klaassen (38) as modified by Thomas and Reed (17). Briefly, at the end of incubation 100% TCA was added to the cells to a final concentration of 12%, and the cell suspension was treated with 0.67% thiobarbituric acid for 20 minutes at 90°C. TBA-reactants formed were measured at 532 nm. Results were expressed in terms of percent of EA-treated cell values.

Mitochondrial Membrane Potential. The distribution of [3 H]-triphenylmethylphosphonium ion (TPMP) between the cardiomyocytes and the incubation solutions was used to determine the collapse of mitochondrial membrane potential. Changes in mitochondrial membrane potential were monitored according to the procedure of Hoeke et al (23). Cells were radiolabeled by incubation with 0.25 μ Ci/ml of [3 H]-TPMP for 1 hr at 37° C. At end of incubation cells were exposed to 150 μ M EA or solvent control for 30-75 min. The results were expressed as percent [3 H]-TPMP released into the culture medium at the end of incubation time.

Measurement of ATP. Following various chemical exposures, ATP levels of cell lysates were determined using the luciferin-luciferase bioluminescent method of Wulf and Doppen (39).

Glyceraldehyde 3-phosphate Dehydrogenase Activity. Myocyte glyceraldehyde 3-phosphate dehydrogenase activity was measured by monitoring the conversion of

3-phosphoglycerol phosphate and NADH to D glyceraldehyde-3-phosphate and NAD⁺, spectrophotometrically according to the method of Birkett et al (13).

Protein Estimation. Certain data as mentioned in the methods was normalized on the basis of per mg protein. All protein values were obtained by the Bradford protein assay (44).

STATISTICAL ANALYSIS: All data were summarized as the mean \pm SD. Significance of the difference between the groups was determined by the 2-tailed Student's t-test.

RESULTS

LDH leakage was used as criterion for assessing the loss of cell viability. As shown in Fig. 1, incubation of cells for 4 hr with increasing concentrations of EA (50-150 μ M) demonstrated a concentration-dependent increase in LDH leakage. There was a concentration-dependent increase in LDH leakage, 57, 78 and 83% with 50, 100 and 150 μ M EA, respectively. There was no significant LDH leakage (6%) in the solvent control even after 4 hr. Further studies conducted with 150 μ M EA for varying amounts of incubation time (7-240 min), exhibited a time-dependent increase in LDH leakage (Fig. 2). LDH leakage was significantly different from the solvent control only after 90 min and beyond, thus permitting us to examine the key biochemical events preceding cell death. At the end of 4 hr incubation, cells treated with 150 μ M EA showed 82% leakage as compared to only 7% for the vehicle control.

The results in Fig. 3 show that there was a rapid depletion of intracellular GSH levels in cells treated with 150 μ M EA. GSH dropped rapidly to 50% of the control levels at 7 min and to less than 1% of controls by 60 min. The status of intracellular protein thiols in the face of an EA challenge was examined over the same time period and illustrated in Fig. 4. We observed a time-dependent depletion in protein thiols, which corresponds to 32% of the control values at 60 minutes. Our data suggests that in EA-induced myocardial cell injury, a significant decrease in GSH precedes the depletion of intracellular protein thiols, and both these events occur well before the onset of LDH leakage is observed.

Intracellular Ca^{2+} levels were monitored by measurement of the Ca^{2+} -dependent conversion of phosphorylase *b* to phosphorylase *a*. As shown in Fig. 5, there was a time-dependent increase in phosphorylase *a* activity when the cells were treated with 150 μ M EA. The elevation of phosphorylase *a* activity, expressed as nmoles Pi / min·mg protein, reached a maximum at 60 min and the value was 124 ± 43 as compared to 18 ± 11 for the controls. This indicates that EA-induced increase in intracellular Ca^{2+} reached a

maximum by 60 min. Again, as with thiol depletion this maximum was reached well before LDH leakage was observed.

The intracellular Ca^{2+} chelators, Quin-2-AM and EGTA-AM were used to assess the importance of EA-induced Ca^{2+} increase in cell injury. These specific intracellular Ca^{2+} chelators were used to attempt buffering the rise in intracellular Ca^{2+} levels, and their effect on subsequent cell injury. Results show LDH leakage after 2 hr incubation with EA (150 μM). Cells preincubated 45 min with Quin-2-AM or EGTA-AM showed leakage of $25 \pm 8\%$ and $11 \pm 2\%$, upon subsequent treatment with EA, respectively (Fig. 6). These data indicate that Quin-2-AM and EGTA-AM decreased LDH leakage, which was 58% at 2 hr, by 57 and 81%, respectively. Pretreatment of cells with just DMSO did not alter EA toxicity (LDH release, $58 \pm 5\%$ as compared to $54 \pm 6\%$). Surprisingly, while both the intracellular Ca^{2+} chelators reduced cell injury, they failed to reduce the magnitude of EA-induced increase in intracellular free Ca^{2+} , based on phosphorylase *a* activity, as shown in Fig.7. Phosphorylase *a* activity in cells treated with 150 μM EA in the presence of DMSO, at the end of 1hr incubation, was found to be 120 ± 27 nmoles Pi / min \cdot mg protein. In comparison, phosphorylase *a* activity of cells preincubated with Quin-2 AM or EGTA AM, preceding an EA challenge was found to be 129 ± 40 and 171 ± 40 nmoles Pi/mg protein \cdot min, respectively. These values were not significantly different from the phosphorylase *a* activity of cells treated with EA, which was 120 ± 27 nmoles Pi / min \cdot mg protein at 1 hr. Furthermore, pretreatment of cells with Quin-2-AM or EGTA-AM, and in the absence of EA, did not alter the phosphorylase *a* activity in comparison to the DMSO control (67 ± 21 and 68 ± 13 , respectively as compared to 50 ± 24 nmoles Pi / min \cdot mg protein at 1 hr). Phosphoinositide hydrolysis initiated by Ca^{2+} -dependent phospholipase *c* activation, was used as an alternative method to verify the observed increase in intracellular Ca^{2+} levels. Fig. 8 shows that phosphoinositide hydrolysis, initiated by Ca^{2+} -dependent phospholipase *c* activation, is 6% in the face of

150 μ M EA challenge. The intracellular Ca^{2+} chelators Quin-2-AM and EGTA-AM have no effect in reducing the extent of phosphoinositide hydrolysis.

Since thiol depletion is known to correlate with oxidative damage, we examined whether EA-induced cell death involved peroxidative damage. Towards this end, cells were treated with 150 μ M EA, in the presence or absence of the antioxidant DPPD (5 μ M), then examined for cell viability at the end of 2 hr incubation. Our results, illustrated in Fig. 9, show that 5 μ M DPPD was capable of reducing damage by 89% for cells treated with 150 μ M EA, as measured by LDH leakage. DPPD treated cells showed $7 \pm 1\%$ leakage as compared to $61 \pm 5\%$ for EA treated cells. The solvent control for DPPD showed no significant amount of leakage at 2 hr. We also assessed the role of DPPD on elevated intracellular Ca^{2+} levels monitored by Ca^{2+} -dependent activation of phosphorylase α . Fig. 10, shows that DPPD had no effect in reducing the elevated levels of intracellular Ca^{2+} in the presence of 150 μ M ethacrynic acid. No changes in phosphorylase α activity was observed in the appropriate controls (65 ± 13 nmoles Pi / min \cdot mg protein for DMSO and 60 ± 9 nmoles Pi / min \cdot mg protein for DPPD) suggesting that there was no interference in our assay by solvents employed. Furthermore, 5 μ M DPPD had no effect on EA-induced depletion of intracellular GSH levels (Table 1).

These observations led us to examine whether EA-induced thiol depletion in myocardial cells led to peroxidative damage. Fig. 11 shows that the antioxidant DPPD, and surprisingly the intracellular Ca^{2+} chelators Quin-2-AM and EGTA-AM, markedly reduced lipid peroxidation, as monitored by the absorbance at 532 nm of 2-thiobarbiturate reaction products. DPPD (5 μ M), Quin-2-AM (120 μ M), and EGTA-AM (120 μ M) significantly reduced lipid peroxidation by 90%, 60%, and 62%, respectively.

The possibility that EA-induced cell injury involved impairment of cellular energy status was assessed by examining the integrity of mitochondrial membrane potential and cellular ATP levels as biochemical indices. Changes in mitochondrial membrane potential

were expressed as the percent [^3H]-TPMP released into the culture medium. Results of previous studies have observed that 90% of [^3H]-TPMP is localized in the mitochondria, and the total cellular content closely corresponds to changes in mitochondrial membrane potential (32). Fig. 12, shows the results of various chemical exposures on the mitochondrial membrane potential, for varying periods of time ranging from 30-75 min. The cells treated with 150 μM EA did not vary significantly from the solvent control values even at 75 min. As a positive control, cells were exposed to an uncoupler of oxidative phosphorylation, CCCP (25 μM). A rapid collapse of mitochondrial membrane potential was observed ($24 \pm 1\%$ at 30 min and $14 \pm 3\%$ at 75 min). ATP levels in the cell lysates were determined for cells treated with 150 μM EA over a time range of 7-75 min. As shown in Table 2, the ATP values for cell treated with EA (46 ± 7 nmoles ATP/mg protein) were different from the control values at 75 min (55 ± 6 nmoles ATP/mg protein).

Previous studies conducted by Birkett *et al* (11), have examined the effect of ethacrynic acid on the cytosolic marker enzyme glyceraldehyde 3-phosphate dehydrogenase (G3PD), and have observed irreversible alkylative damage of the protein thiol groups. Fig. 13 shows the results of our studies conducted on this marker enzyme. G3PD activity was expressed as $\mu\text{moles NADH/min-mg protein}$ and measured at the end of 1 hr incubation with various chemical exposures. Cells were treated with 150 μM EA in the presence or absence of 120 μM Quin-2-AM, 120 μM EGTA-AM or 5 μM DPPD. Our studies demonstrates that ethacrynic acid diminishes the enzyme activity to 30% of the control values, whereas the intracellular Ca^{2+} chelator EGTA-AM, and the antioxidant DPPD had no protective effect, but surprisingly Quin-2-AM provided 88% protection to the enzyme activity.

Figure 1. *Concentration dependent effect of EA on cell viability in cultured cardiomyocytes.* Myocytes were incubated at 37° C in the presence of a range of EA concentrations (0-150 μ M) in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol (v/v) and buffered to pH 7.4 with 25 mM Hepes. Cell viability was determined by leakage of LDH after 4 hr incubation with EA. Values represent the mean \pm SD of duplicate assays, from 3 separate myocyte preparations.

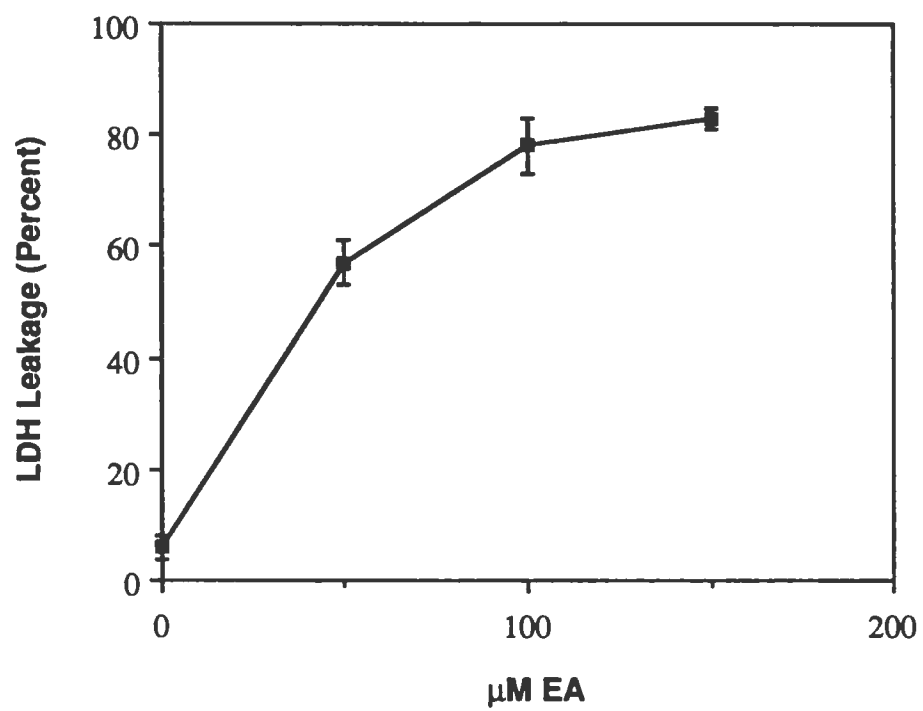


Figure 2. *Time dependence of the effect of 150 μ M EA on cardiomyocyte cell viability.* Myocytes were incubated at 37° C in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol (v/v) and buffered to pH 7.4 with 25 mM Hepes, in the absence (\square) or presence (\blacksquare) of 150 μ M EA. Values represent the mean \pm SD of measurements from 3-12 separate preparations. Differences between EA and control cells were significant at $p < 0.01$ (*).

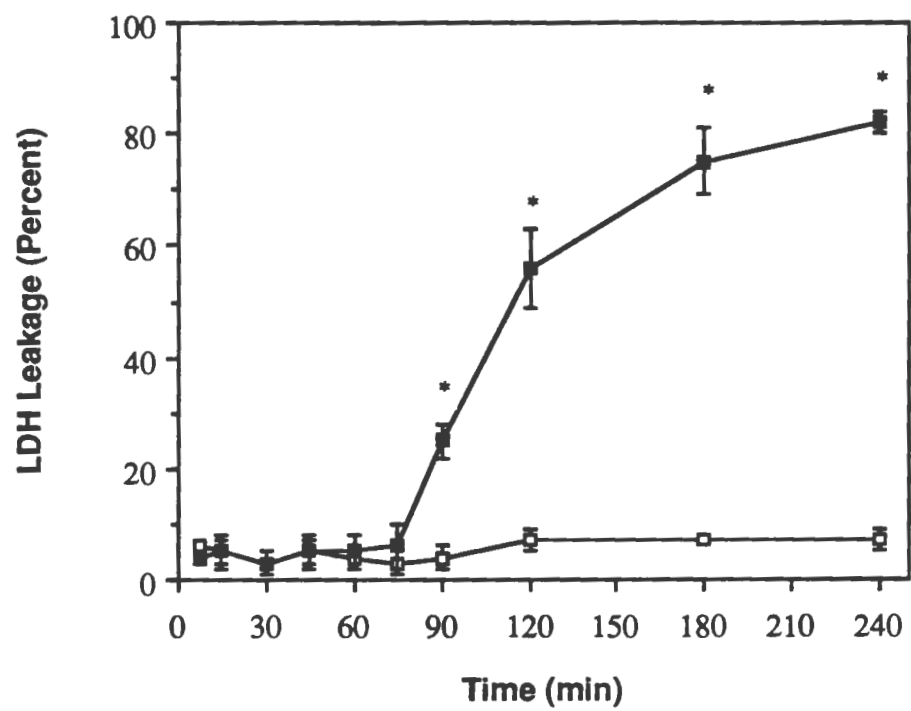


Figure 3. *Time dependence of the effect of 150 μ M EA on cardiomyocyte glutathione (GSH) levels.* Myocytes were incubated at 37° C in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol (v/v) and buffered to pH 7.4 with 25 mM Hepes, in the absence (\square) or presence (\blacksquare) of 150 μ M EA. At times indicated, GSH levels were determined by HPLC method of Reed et al (37). Values represent the mean \pm SD of measurements from 3 separate myocyte preparations. Differences between EA treated cells and control cells are significant at $p < 0.01$ (*).

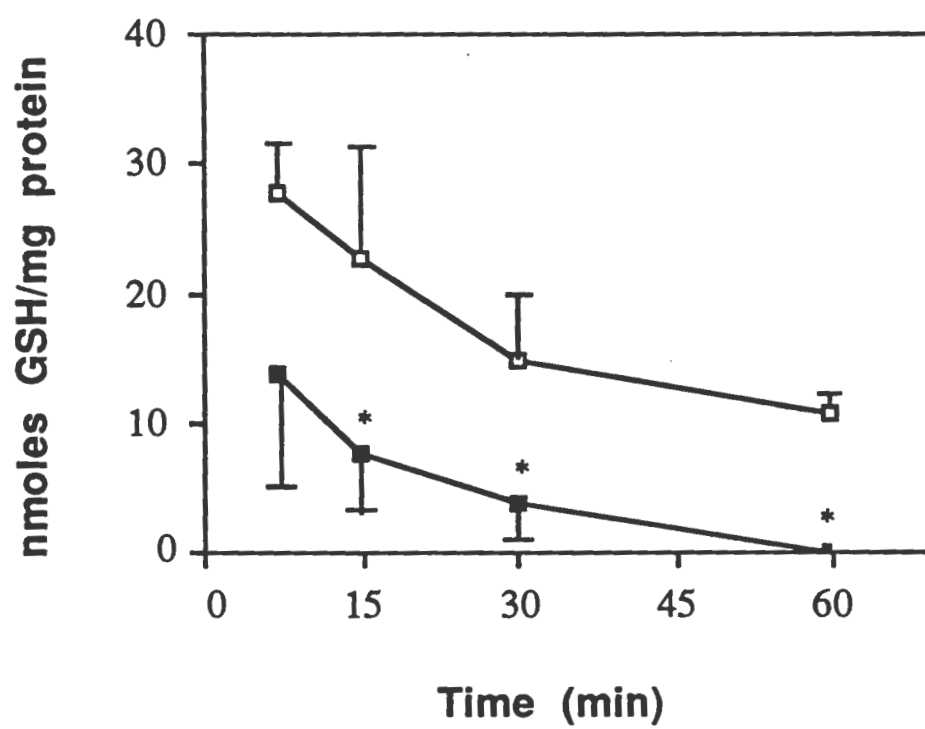


Figure 4. *Time dependence of the effect of 150 μ M EA on cardiomyocyte protein thiol levels.* Myocytes were incubated at 37° C in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol (v/v) and buffered to pH 7.4 with 25 mM Hepes, in the absence (\square) or presence (\blacksquare) of 150 μ M EA. At times indicated the content of myocyte protein thiols was determined. Values represent the mean \pm SD of measurements from 7 separate myocyte separations. Differences between EA treated cells and control cells are significant at $p < 0.05$ (*) and $p < 0.01$ (**).

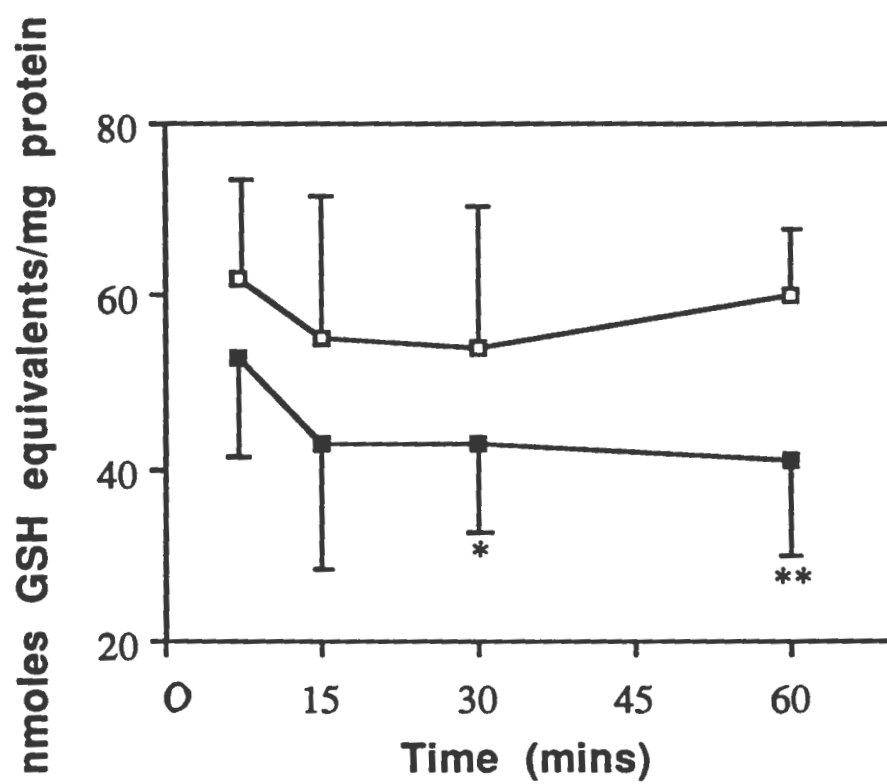


Figure 5. *Time dependence of the effect of 150 μ M EA on cardiomyocyte phosphorylase a activity.* Myocytes were incubated at 37° C in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol (v/v) and buffered to pH 7.4 with 25 mM Hepes, in the absence (\square) or presence (\blacksquare) of 150 μ M EA. At times indicated the phosphorylase a activity was determined. Values represent the mean \pm SD, at 7, 15, 45, and 75 min (n = 3), at 30 min (n = 6), and at 60 min (n = 9). Differences between EA treated cells and control cells are significant at $p < 0.01$ (*).

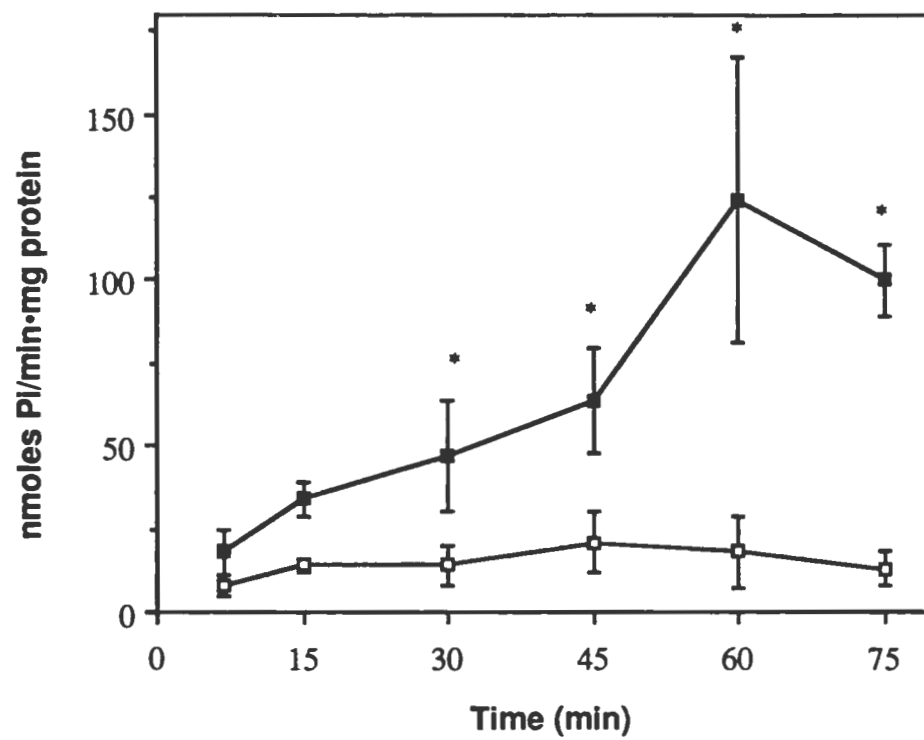


Figure 6. *Effect of intracellular Ca^{2+} chelators, Quin-2 -AM and EGTA-AM, on EA-induced cell injury in cultured cardiomyocytes.* Cardiomyocytes were preincubated with 120 μ M Quin-2-AM, EGTA-AM, or 0.5% DMSO (v/v) for 45 min. At that time the myocyte cultures were rinsed twice with Hank's balanced salt solution (1.2 mM Ca^{2+}) containing 25 mM Hepes, pH 7.4 at 37°C. Myocytes were then incubated in Hank's balanced salt solution, 0.3% ethanol, in the absence (\square) or presence (\blacksquare) of 150 μ M EA. At the end of 2 hr incubation, cell viability was determined based on the leakage of LDH. Values represent the mean \pm SD of 3-7 separate cardiomyocyte preparations. Differences between EA treated cells and control cells, in the absence or presence of EGTA-AM and Quin-2-AM are significant at $p < 0.01$ (*).

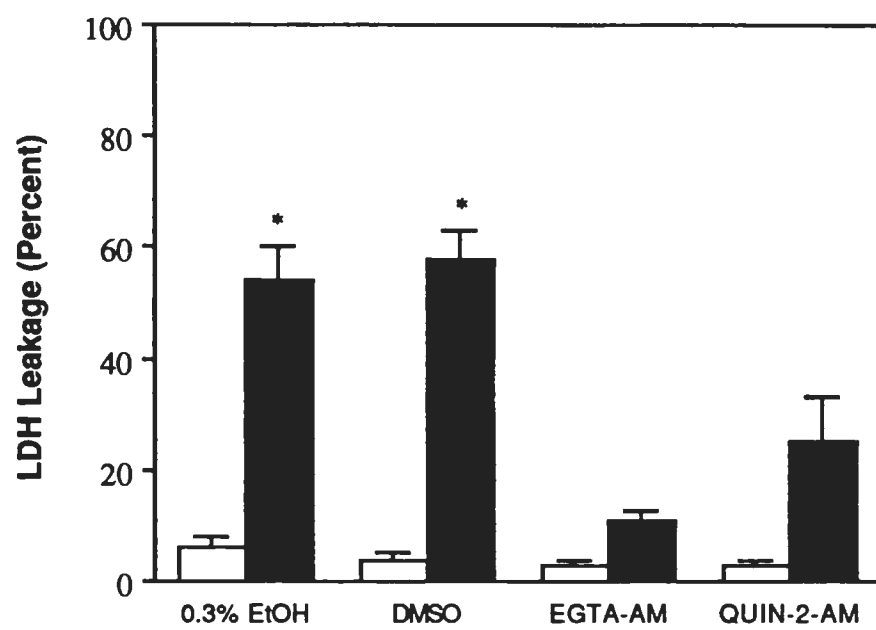


Figure 7. *Effect of intracellular Ca^{2+} chelators, Quin-2 -AM and EGTA-AM, on EA-induced increase of phosphorylase a activity in cultured cardiomyocytes.* Cardiomyocytes were preincubated with 120 μ M Quin-2-AM or EGTA-AM or 0.5% DMSO (v/v) for 45 min. Cultures were rinsed twice with Hank's balanced salt solution (1.2 mM Ca^{2+}) containing 25 mM Hepes, pH 7.4. Myocytes were then incubated in Hank's balanced salt solution, 0.3% ethanol, in the absence (\square) or presence (\blacksquare) of 150 μ M EA. At the end of 1 hr incubation at 37°C the myocyte phosphorylase a activity was determined. Values represent the mean \pm SD of 3-7 separate cardiomyocyte preparations. Differences between EA treated cells and controls cells are significant at $p < 0.01$ (*), but there is no significant difference between cells treated with and without EGTA-AM and Quin-2-AM.

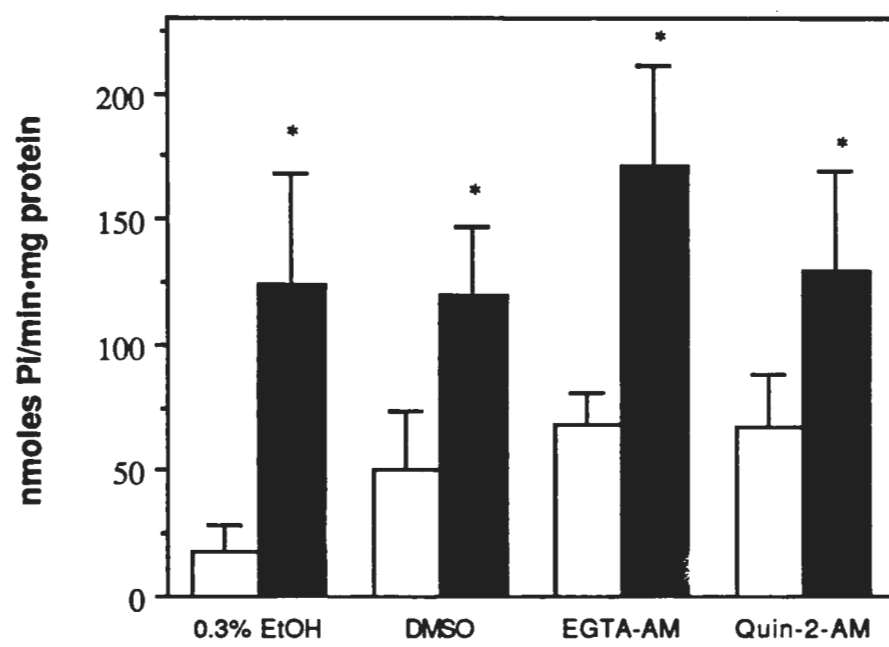


Figure 8. *Effect of intracellular Ca^{2+} chelators, EGTA-AM and Quin-2-AM, and antioxidant DPPD on EA-induced phosphoinositide hydrolysis by Ca^{2+} -activated phospholipase c.* Cells were radiolabelled by the incorporation of 2 μ Ci/ml of [2- 3 H]-myo-inositol for 24 hr at 37°C. At that time radiolabelled media was removed and cardiomyocytes were preincubated with 120 μ M Quin-2-AM, EGTA-AM or 0.5% DMSO (v/v) for 45 min. At that time cells were rinsed twice with Hank's balanced salt solution (1.2 mM Ca^{2+}) containing 25 mM Hepes, pH 7.4 at 37°C. Myocytes were then incubated in Hank's balanced salt solution, 0.3% ethanol, at 37°C for 1 hr, in the absence or presence of 150 μ M EA. Results were expressed as the difference between control and EA values. Separate cardiomyocyte preparations were also treated with 5 μ M DPPD concurrently with or without 150 μ M EA, in Hank's balance salt solution, 0.3% ethanol (v/v) and 0.4% DMSO (v/v). Values represent the mean \pm sd of of measurements from 4 separate myocyte preparations. There was no significant difference between cells treated with or without EGTA-AM, Quin-2-AM, or DPPD.

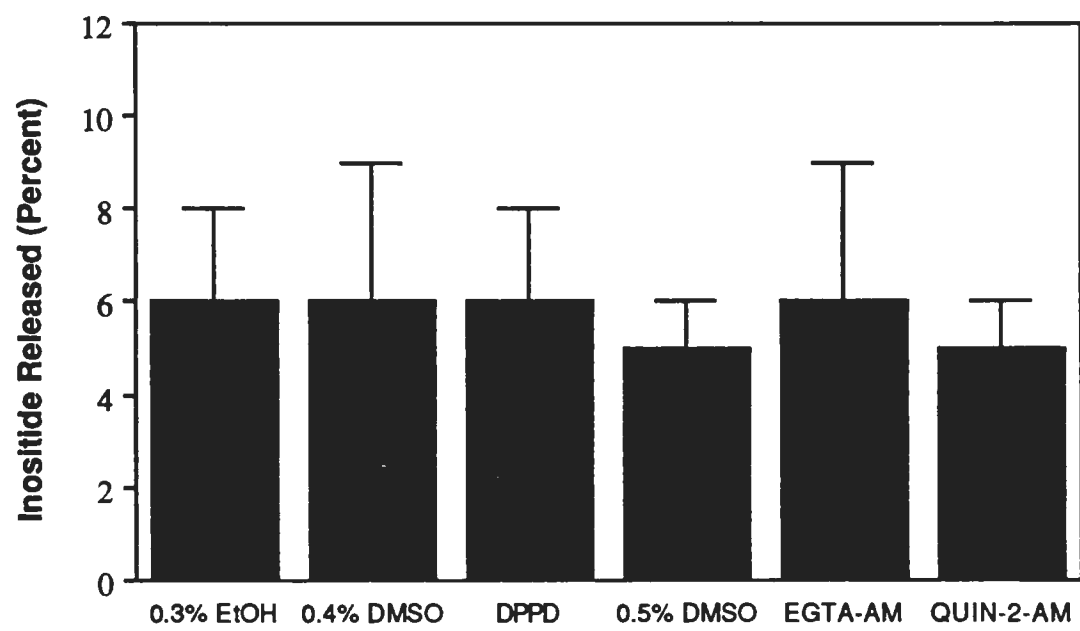


Figure 9. *Effect of antioxidant DPPD, on EA-induced cell injury in cultured cardiomyocytes.* Myocytes were incubated in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol (v/v), or 0.3% ethanol (v/v) and 0.5% DMSO (v/v), and buffered to pH 7.4 with 25 mM Hepes at 37° C, in the absence (□) or presence (■) of 150 μM EA, with or without 5 μM DPPD. At the end of 2 hr incubation at 37°C cell viability was determined based on the leakage of LDH. Values represent the mean \pm sd of 4-9 separate cardiomyocyte preparations. Differences between EA treatments with and without DPPD are significant at $p < 0.01$ (*).

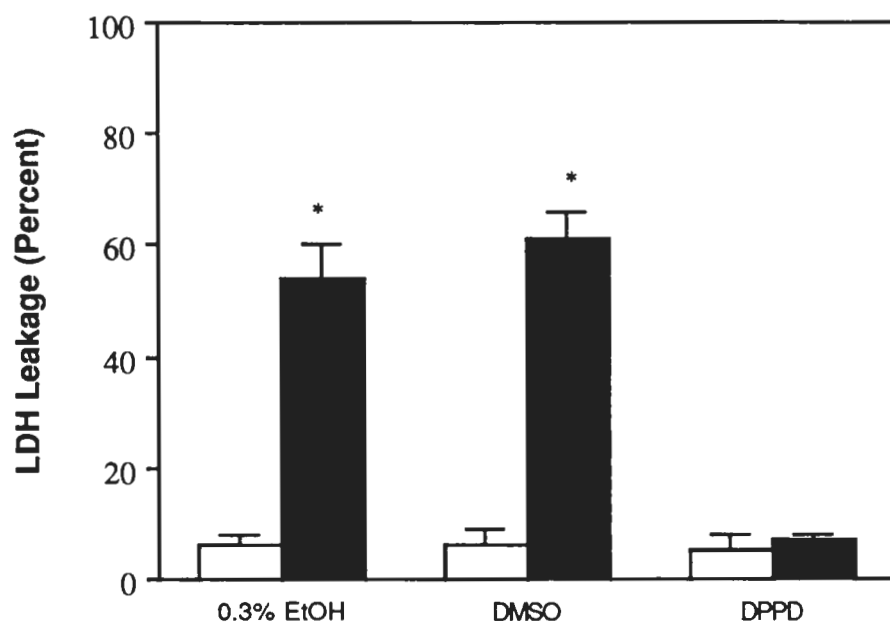


Figure 10. *Effect of antioxidant DPPD, on EA-induced increase of phosphorylase a activity in cultured cardiomyocytes.* Myocytes were incubated at 37° C in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol (v/v), or 0.3% ethanol (v/v) and 0.5% DMSO (v/v), and buffered to pH 7.4 with 25 mM Hepes, in the absence (\square) or presence (\blacksquare) of 150 μM EA. At the end of 1 hr incubation at 37°C the myocyte phosphorylase activity was determined. Values represent the mean \pm SD of 4 separate cardiomyocyte preparations. Differences between EA treated cells and controls cells are significant at $p < 0.01$ (*), but there is no significant difference between cells treated with and without DPPD.

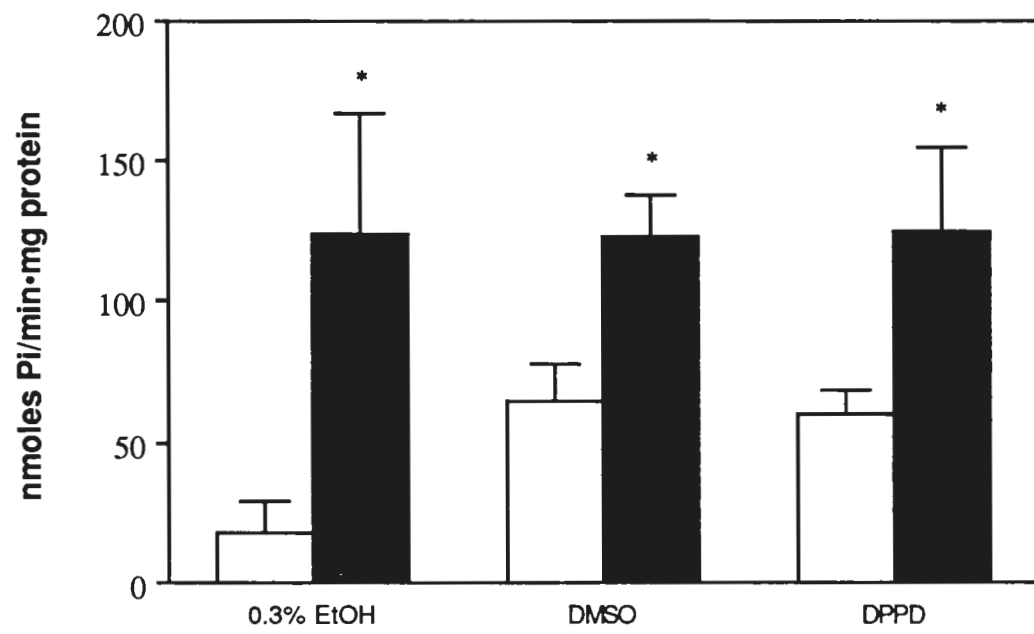


Figure 11. *Lipid peroxidation in cardiomyocytes.* Cardiomyocytes were preincubated with 120 μ M Quin-2-AM, EGTA-AM for 45 min. At that time cultures were rinsed twice with Hank's balanced salt solution containing 1.2 mM Ca^{2+} and buffered to pH 7.4 with 25 mM Hepes. Myocytes were then incubated in the absence (\square) or presence (\blacksquare) of 150 μ M EA in Hank's balanced salt solution, 0.3% ethanol, at 37°C for 4 hr. Separate cardiomyocyte preparations were also concurrently treated with 5 μ M DPPD in Hank's balanced salt solution, 0.3% ethanol (v/v) and 0.4% DMSO (v/v), with or without 150 μ M EA. TBA reactants were measured at the end of 4 hr incubation. Values represent the mean \pm SD of 4 separate myocyte preparations. Differences between EA treated cells and control cells, and cells with or without EGTA-AM, Quin-2-AM, and DPPD, are significant at $p < 0.01$ (*).

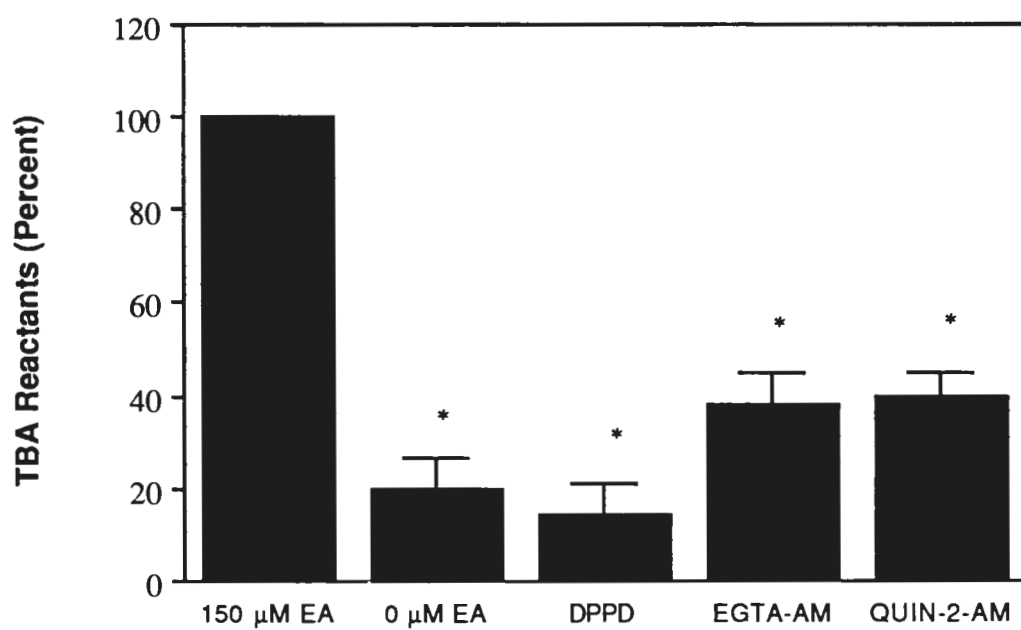


Figure 12. *Effect of 150 μ M EA on cardiomyocyte mitochondrial membrane potential.* Cells were radiolabelled with 0.25 μ Ci/ml of [3 H]-TPMP for 1hr at 37 °C. At that time cultures were rinsed twice with Hank's balanced salt solution (1.2 mM Ca^{2+}) containing 25 mM Hepes, pH 7.4 at 37°C. Myocytes were then incubated in Hank's balanced salt solution, 0.3% ethanol, for 30-75 min at 37°C, in the absence (\square) or presence (\blacksquare) of 150 μ M EA. Myocytes were also incubated with 25 μ M CCCP (\blacktriangle), 0.5% DMSO (v/v), as a positive control. Values represent the mean \pm SD of n = 7 (0 μ M EA, 150 μ M EA), and n = 6 (25 μ M CCCP). Differences between EA treated cells and controls cells are significant at $p < 0.01$ (*) and between CCCP treated cells and control cells.

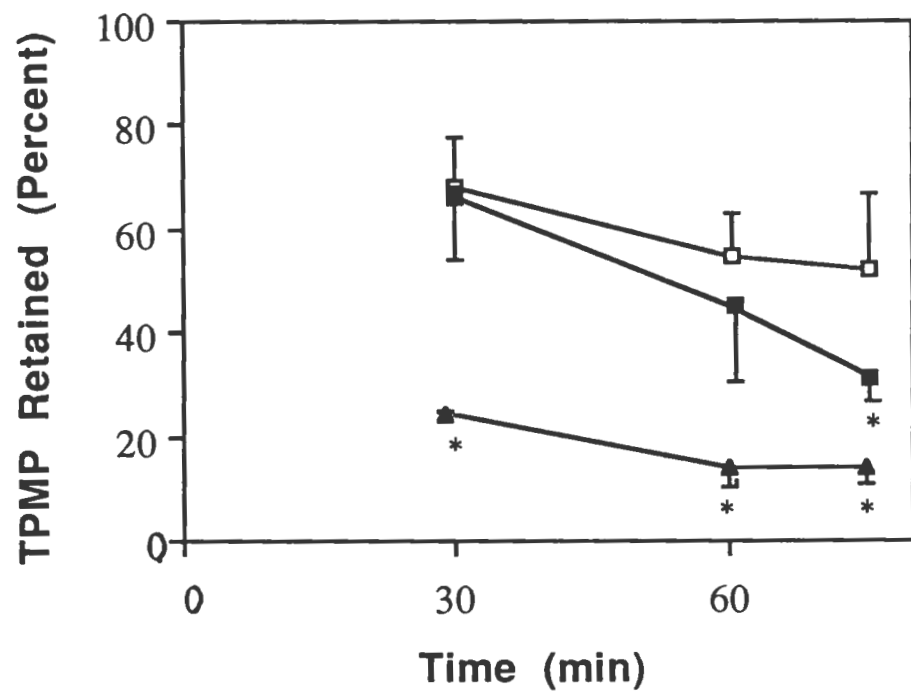


Figure 13. *Effect of intracellular Ca^{2+} chelators, EGTA-AM and Quin-2-AM, and antioxidant DPPD on EA-induced inactivation of glyceraldehyde 3-phosphare dehydrogenase activity.* Cardiomyocytes were preincubated with 120 μM Quin-2-AM, EGTA-AM or 0.4-0.6% DMSO for 45 min. At that time cultures were rinsed twice with Hank's balanced salt solution (1.2 mM Ca^{2+}), containing 25 mM Hepes, pH 7.4 at 37°C. Myocytes were then incubated in Hank's balanced salt solution, 0.3% ethanol, at 37° C for 1 hr in the absence (\square) or presence (\blacksquare) of 150 μM EA . Separate cardiomyocyte preparations were also treated with 5 μM DPPD concurrently with or without 150 μM EA, in Hank's balance salt solution, 0.3% ethanol (v/v) and 0.4% DMSO (v/v). Values represent the mean \pm SD of 4 separate myocyte preparations. Differences between EA treated cells and control cells are significant at $p < 0.01$ (*).

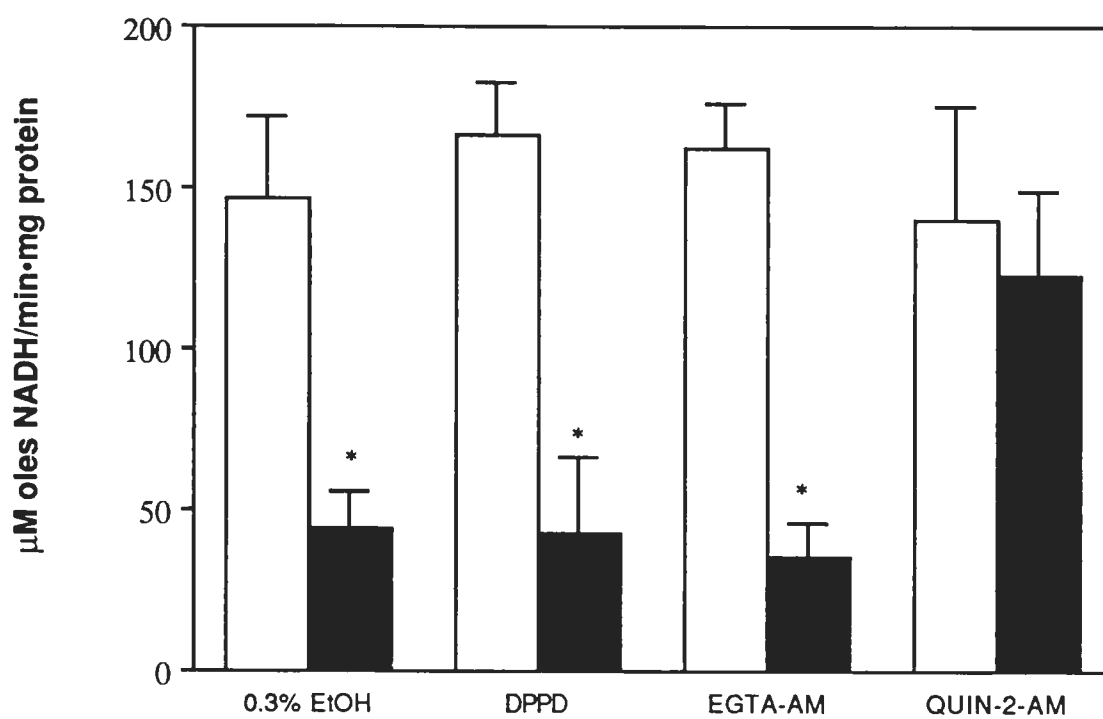


Table 1. *Effect of DPPD on EA- induced cardiomyocyte GSH depletion.*

Treatment	nmols GSH/mg protein
0 μ M EA	
(-) DPPD	19 \pm 3
(+) DPPD	17 \pm 4
150 μ M EA	
(-) DPPD	3 \pm 2*
(+) DPPD	3 \pm 1*

Myocytes were incubated in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol, and buffered with 25 mM Hepes to pH 7.4 at 37° C, in the absence or presence of 150 μ M EA, with or without 5 μ M DPPD. At the end of 30 min incubation, GSH levels were determined by HPLC method of Reed et al (37). Values represent the mean \pm SD of measurements from 3 separate myocyte separations. Differences between EA treated cells and control cells are significant at $p < 0.01$ (*).

Table 2. *Time-dependent effect of 150 μ M EA on cardiomyocyte ATP levels.*

Time(mins)	nmoles ATP/mg protein	
	Control	150 μ M EA
7	52 \pm 5	53 \pm 11
15	52 \pm 5	53 \pm 8
30	52 \pm 7	55 \pm 6
60	55 \pm 3	52 \pm 2
75	55 \pm 6	46 \pm 7*

Myocytes were incubated in Hank's balanced salt solution containing 1.2 mM Ca^{2+} , 0.3% ethanol, and buffered with 25 mM Hepes to pH 7.4 at 37° C, in the absence or presence of 150 μ M EA. At times indicated ATP levels were determined by the luciferin-luciferase method as mentioned in the method section. Values represent the mean \pm SD of measurements from 4 separate myocyte preparations. Differences between EA treated cells and control cells are significant at $p < 0.05$ (*).

DISCUSSION

Using exposure to EA as a model of chemically-induced cytotoxicity, we examined the relationship between intracellular thiol status, Ca^{2+} homeostasis, and myocardial cell death. The maintenance of intracellular GSH has been suggested to play a pivotal role in the maintenance of cell viability (1, 2, 4). Results of previous studies suggest that depletion of GSH levels to less than 10-15% of initial levels is generally observed to correlate with the loss of cell viability (6). These results have been interpreted to suggest that the extent of cell death following the chemical depletion of GSH corresponds to depletion of the mitochondrial pool, which comprises 15% of the total intracellular GSH (4). We observed a rapid depletion of intracellular GSH levels to less than 1% within 1 hr in the presence of 150 μM EA. These data demonstrate that the EA challenge in our model system was of sufficient magnitude to deplete almost all of the intracellular GSH, including the mitochondrial pool.

Recent studies have led to the suggestion that the alteration of protein thiols in concert with the depletion of intracellular GSH may be responsible for the loss of protection against a chemically-induced oxidative stress and subsequent loss of cell viability (43). EA has been effectively used to deplete intracellular thiols in isolated rat hepatocytes (3). Modification of these protein thiol groups may limit the enzymatic capacity of many critical enzymes. Such enzymes include sarcoplasmic reticular and sarcolemmal Ca^{2+} -ATPases, and modification of these enzymes result in the perturbation of intracellular Ca^{2+} homeostasis preceding cell injury (10, 11). Results described herein, demonstrate a time-dependent depletion of protein thiols (30% depletion by 1 hr), which follows the depletion of intracellular GSH. This pool of depleted protein thiols may include thiol containing enzymes responsible for maintaining intracellular Ca^{2+} homeostasis. Thus this alteration of critical thiol groups may be linked to the perturbation of Ca^{2+} homeostasis,

and the resultant elevation of cytosolic free Ca^{2+} may contribute to cell death.

Given the proposed relationship between thiol status and Ca^{2+} homeostasis and the possible significance of these events to cell viability, we examined intracellular Ca^{2+} homeostasis in the face of an EA challenge. A time and concentration-dependent increase in cytosolic free Ca^{2+} levels was observed in myocardial cells, as measured by the elevation of phosphorylase *a* activity. The elevation of intracellular Ca^{2+} followed the depletion of intracellular GSH and protein thiols, and preceded the onset of cell death. The temporal relationship we observed suggests that an EA challenge may alter thiol groups critical to the regulation of intracellular Ca^{2+} , resulting in elevated cytosolic Ca^{2+} . This perturbation of Ca^{2+} homeostasis may contribute to cell injury due to Ca^{2+} -activated processes.

This temporal relationship suggested that intracellular thiol depletion may be linked to the perturbation of Ca^{2+} homeostasis that preceded cell death. We next attempted to determine if the observed Ca^{2+} increase was responsible for the cell injury. Toward this end, we tested the effect of two specific intracellular Ca^{2+} chelators, Quin-2-AM and EGTA-AM, on EA toxicity. While both the chelators protected the cells, contrary to expectations, they did not do so by lowering Ca^{2+} . These results do not support interpretations from other studies that elevated Ca^{2+} levels are a primary cause of cell death.

Besides its proposed importance in Ca^{2+} homeostasis, GSH is a major intracellular antioxidant. The extent of GSH depletion caused by EA treatment suggested a possible role of peroxidative damage in our system of chemically-induced injury. Indeed, our results indicate that EA-induced thiol depletion results in lipid peroxidation. This rapid depletion of intracellular GSH may incapacitate the effective reduction of free radicals and the resultant generation of partially reduced oxygen species may initiate an iron-dependent mechanism of peroxidative damage to the cellular membrane (22). The antioxidant DPPD

was used to investigate the importance of the loss of GSH antioxidant protection in a situation where GSH is depleted due to its alkylation by EA. We also observed the effect of DPPD on EA-induced lipid peroxidation, in the absence of GSH as an antioxidant. DPPD protected cardiomyocytes in the face of an EA challenge that virtually eliminated intracellular GSH, and also reduced the subsequent lipid peroxidation by 90%. Furthermore, the antioxidant DPPD conferred 89% protection against EA-induced cell death, but had no effect on the elevated phosphorylase *a* activity. The protective property of DPPD may be attributed exclusively to its ability to prevent lipid peroxidation (9). These data showed that elevated cytosolic Ca^{2+} alone had no effect on cell viability over the time course of our experiments. However lipid peroxidation appeared to be a key factor in the onset of cell death.

Treatment of the cells with the Ca^{2+} chelators Quin-2-AM and EGTA-AM, markedly decreased lipid peroxidation by 60% and 62%, respectively. The ability of iron to undergo redox reactions linked to single-electron cycling is well known. This Fenton-type of reaction which generates free radicals, may possibly interact with the cellular membrane and cause lipid peroxidation. The protective action of the Ca^{2+} chelators may be more complex to explain. One possibility is that chelation of endogenous iron may protect against lipid peroxidation, which would inhibit a Fenton-type reaction (42).

While our data supports a primary role for peroxidative damage in EA-induced cell death, it was also possible that EA treatment might compromise cellular energy status. Our results demonstrate that EA inhibits G3PD activity, thus demonstrating a potential to inhibit the enzymatic activity of other thiol containing enzymes. This inhibition of such thiol containing enzymes may, in turn, lead to an alteration in normal cellular ATP production. Results of previous studies conducted on myocardial cells have demonstrated that ATP levels may be depleted to 50% of their initial levels without any corresponding

decrease in cell viability (45). We monitored the mitochondrial membrane potential and cellular ATP levels, as an important indicator of cellular energy status. Our data indicate that ATP levels did not decrease appreciably (< 16% by 75 min) and mitochondrial membrane potential is not significantly impaired during EA exposure. These results suggest that the EA-induced alteration of cellular energy status does not play a major role in the onset of cell injury in this model system.

Taken collectively, the results of this study suggest that while elevated cytosolic free Ca^{2+} may contribute to the cell injury from thiol depletion, we observed, it alone is not the cause of cell death; oxidative damage is a primary requisite. The fact that EA by itself does not generate any free radicals, leads to further speculation regarding the source of oxidative damage in our model system. This study also demonstrates the utility of EA-treated myocytes as a model to study the biochemical events involved in oxidative stress.

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